Volume 220, number 2, 337-341 FEB 05020 August 1987

Collagen type IX: evidence for covalent linkages to type II collagen in cartilage

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Received 30 June 1987

A major site of pyridinoline cross-linking in bovine type IX collagen was traced to a tryptic peptide derived from one of the molecule's HMW chains. This peptide gave two amino acid sequences (in 2/1 ratio) consistent with it being a three-chained structure. The major sequence matched exactly that of the C-telopeptide of type II collagen from the same tissue. A second HMW chain that contained pyridinoline cross-links also gave two amino-terminal sequences, one from its own amino terminus, the other matching exactly the N-telopeptide cross-linking sequence of type II collagen. We conclude that type IX collagen molecules are covalently cross-linked in cartilage to molecules of type II collagen, probably at fibril surfaces.

Collagen; Cross-linking; Amino acid sequence; (Bovine cartilage)

1. INTRODUCTION

Hyaline cartilage contains at least three tissue-specific collagens, types II, IX and XI ($1\alpha 2\alpha 3\alpha$) [1]. Type II forms the tissue's fibrillar network, accounting for over 90% of the total collagen. Type IX collagen is an unusual molecule that consists of three triple-helical domains joined by short non-triple-helical sequences [2-7]. It accounts for 1-2% of the collagen in adult hyaline cartilage and up to 10% in fetal cartilage. Pepsin solubilizes type IX collagen in the form of two main, pepsin-resistant fragments, HMW and LMW [3]. In chick cartilage one or two chondroitin sulfate or dermatan sulfate chains are attached per type IX collagen molecule, which therefore is also a type of proteoglycan [8-11]. Type IX collagen from bovine articular cartilage contains hydroxypyridinium (pyridinoline) cross-linking residues at a higher molar concentration than found in type II collagen [12], making it one of the richest sources of these collagen-specific trivalent cross-links [13]. In the analyses described here, peptides were isolated and structurally characterized that originated from the hydroxypyridinium cross-linking sites in bovine type IX collagen.

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Abbreviations: HP, hydroxylysyl pyridinoline; LP, lysyl pyridinoline; HMW and LMW, triple-helical segments of the type IX collagen molecule released by pepsin; C-telopeptide and N-telopeptide, short sequences that form the amino- and carboxy-ends of types I, II and III collagen chains; HPLC, high-performance liquid chromatography

2. MATERIALS AND METHODS

Type IX collagen was extracted by pepsin from 2 year-steer articular cartilage and purified by salt precipitation [12]. The isolated protein was free of other collagens on SDS-polyacrylamide electrophoresis. After reduction and carboxymethylation of cysteines, the HMW and LMW chain fragments of the type IX molecule were resolved by molecular-sieve column chromatography on 8% agarose (170 cm × 1.5 cm; Bio-Rad A1.5m,

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00145793/87/$3.50

200–400 mesh) eluted with 1 M $CaCl_2$, 0.05 M Tris-HCl, pH 7.5 [14]. Individual chains from the isolated HMW domain were resolved by reverse-phase HPLC on a macroporous C4-column (25 cm × 4.6 mm; Vydac 214TP54) eluted by a gradient of acetonitrile:*n*-propanol (3:1, v/v) in aqueous 0.1% (v/v) trifluoroacetic acid. Tryptic peptides prepared from total HMW or from its individual chains were resolved by a combination of molecular-sieve column chromatography on Bio-Gel P-10 (90 cm × 2.5 cm; 200–400 mesh; Bio-Rad) eluted in 10% acetic acid, ion-exchange HPLC (DEAE5PW, 7.5 cm × 7.5 mm, Bio-Rad) with a linear NaCl-gradient in 0.02 M Tris-HCl, pH 7.5, and reverse-phase HPLC on a C18 column (25 cm × 4.6 mm; Vydac 218TP54) eluted with an acetonitrile gradient in aqueous 0.01 M *n*-heptafluorobutyric acid. Column effluents were monitored for polypeptide absorbance at 220 nm and specifically for the fluorescence of the hydroxypyridinium cross-linking amino acids (emission at 395 nm; excitation at 297 nm for acid pH or 330 nm for neutral pH [15]).

Amino acid compositions were determined after hydrolysis in 6 M HCl (24 h at 108°C) using pre-column derivatization with phenylisothiocyanate [16] and reverse-phase chromatography on a Brownlee Ultrasphere ODS column (25 cm × 4.6 mm) using a Waters HPLC system. Peptides and chains were subjected to amino-terminal sequence analysis on a Beckman 890B or 890M sequencer using polybrene [17], and identifying the PTH amino acids by two different HPLC methods [18,19].

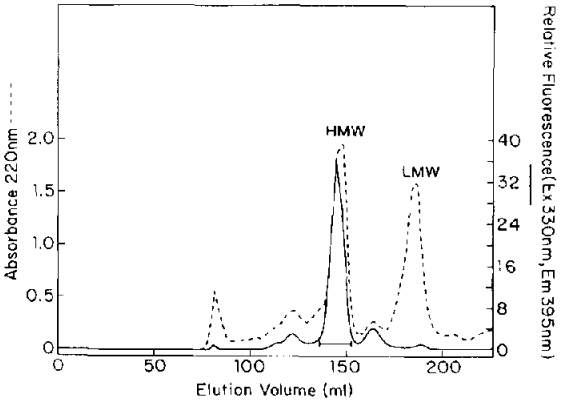

Fig.1. Molecular-sieve column chromatography of the pepsin-generated domains of bovine type IX collagen on 8% agarose (Bio-Rad A1-5m). The fluorescent pyridinoline cross-linking residues were recovered in the HMW fraction.

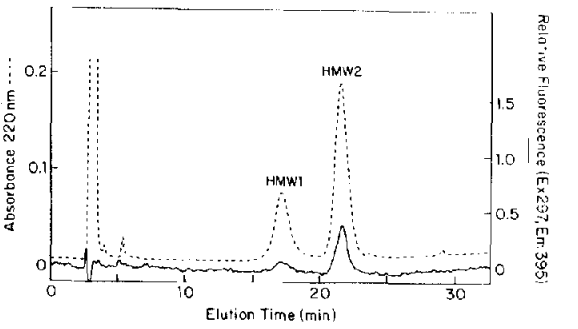

Fig.2. Reverse-phase HPLC (C-4 column) to resolve two chain fractions from HMW isolated by agarose chromatography (pooled as in fig.1).

3. RESULTS

Fig.1 shows the resolution by agarose column chromatography of pepsin-extracted and purified type IX collagen into its HMW and LMW chain fragments. All the cross-link-specific fluorescence was associated with HMW, as found by gel electrophoresis [12].

The HMW fraction was resolved into two protein peaks by C-4 reverse-phase chromatography

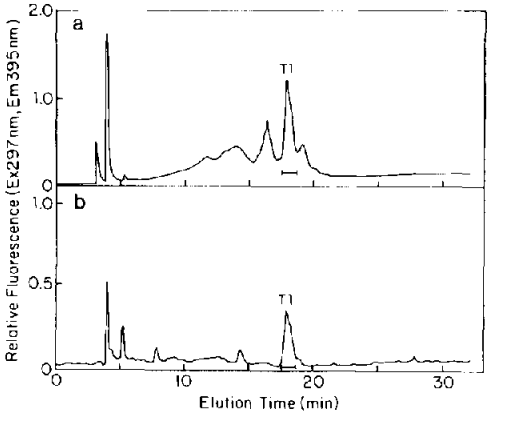

Fig.3. Reverse-phase HPLC (C-18 column) as the final purification step for the main HP-containing tryptic peptide (T1) from type IX collagen. (a) Fluorescent cross-linked peptides resolved from a trypsin digest of total HMW (fig.1). (b) The fluorescent cross-linked peptide derived by trypsin from HMW2 (fig.2).

Table 1

Amino acid composition of tryptic peptide T1 derived from the main pyridinoline cross-linking site in type IX collagen

	Residues/peptide
Hyp	3 (3.2)
Asp	2 (1.6)
Glu	5 (5.0)
Pro	4 (3.9)
Gly	9 (9.0)
Ala	1 (1.3)
Leu	3 (2.6)
His	1 (1.0)
Arg	2 (2.0)
HP + LP[a]	1 (0.7)
Total	31

[a] Quantified on reverse-phase HPLC by 3-pyridinol fluorescence [23]. The ratio of HP to LP was about 4 to 1

(HMW1 and HMW2, fig.2), with 80% of the pyridinoline fluorescence in HMW2. A trypsin digest of the whole HMW fraction revealed one major fluorescent peptide on reverse-phase HPLC (T1, fig.3a). The same fluorescent peptide was recovered from a trypsin digest of purified HMW2 (fig.3b).

The amino acid composition of the cross-linked tryptic peptide (table 1) indicates 31 residues. Amino-terminal sequence analysis gave a major sequence and a minor sequence indicating the partial structure shown in fig.4a. In comparison, fig.4b shows the structure of one of two main pyridinoline cross-linked peptides isolated, and sequenced by similar methods, from a trypsin digest of pepsin-solubilized and purified bovine type II collagen from the same tissue (manuscript in preparation). The results indicate that two peptide arms of the type IX-derived cross-linked peptide and two peptide arms of the type II-derived cross-linked peptide have the same sequence. This sequence comes from the C-telopeptide cross-linking domain of the α1(II)-chain by homology to the human and chick gene sequences [20].

The cross-linking residue could not be directly identified as a PTH derivative, its sites only inferred by blanks in the two running sequences. The

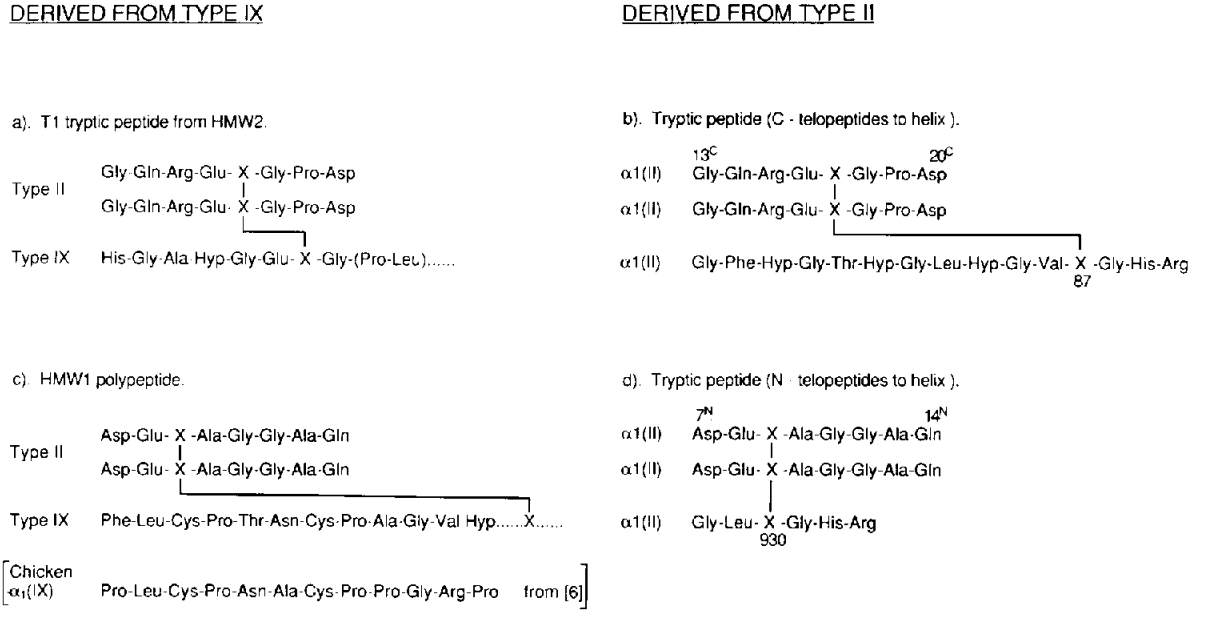

Fig.4. Amino-terminal structures deduced from the Edman degradation of cross-linked peptides purified from types IX and II collagens of bovine articular cartilage. (a). T1 tryptic peptide (fig.3) from the main cross-linking site in type IX collagen (HMW2 chain fraction). (b) The major cross-linked tryptic peptide from type II collagen (C-telopeptide site). (c) Deduced amino-terminal sequences for the HMW1 chain fraction (fig.2). (d) The major cross-linked tryptic peptide from type II collagen (N-telopeptide site). The amino termini of the type II telopeptide sequences are created by the pepsin-digestion needed to solubilize the collagens.

3-pyridinol ring structure, and hence the potentially detectable fluorescence of HP and LP (the two structural forms of pyridinoline cross-linking residue), was destroyed by the cycles with 0.1 M Quadrol, pH 9.2, at 52°C in the spinning cup (the half-life of HP was about 40 min under these conditions).

Direct amino-terminal sequence analysis on the second resolved popypeptide from the HMW triple-helix (HMW1, fig.2), which gave a single band on SDS-polyacrylamide electrophoresis (not shown), again revealed two sequences which are interpreted as shown in fig.4c. One of the sequences is identical to that found on two arms of the other major cross-linked peptide derived from pure type II collagen (fig.4d), which comes from the N-telopeptide cross-linking domain of the α1(II)-chain by homology to the rat gene sequence [21]. This second HMW chain, therefore, apparently also contains a site of cross-linking which can link to the N-telopeptide domain of type II collagen. Its deduced amino-terminal sequence shows similarities (fig.4c), notably in the placement of cysteines, to the chick α1(IX)-chain over the sequence that spans the junction of the non-collagenous (NC3) and the main collagenous (COL2) domains of HMW [6].

4. DISCUSSION

The results provide direct evidence that molecules of type IX collagen are covelantly cross-linked in cartilage to molecules of type II collagen through the hydroxylysine-aldehyde mediated mechanism [13]. The sequence data imply that telopeptide aldehydes on type II collagen can link to the type IX collagen molecule at one or more sites on at least two chains of its COL2 triple-helical domain [7]. The mature cross-linking structures are trivalent pyridinoline residues which are also the main aldehyde-mediated intermolecular cross-links within type II collagen fibrils. Their mechanism of formation is uncertain, for example whether the two telopeptide arms on each pyridinoline residue derive from one or two type II collagen molecules. In the T1 tryptic peptide, which consists of two type II C-telopeptides linked to a sequence from the type IX triple-helix (COL2 domain [7]), it is not yet certain which α-chain this latter peptide derives from in terms of the α1(IX)-, α2(IX)- and α3(IX)-chains of chick type IX collagen [10].

The sequencing results on the HMW1 polypeptide (the lesser source of pyridinoline cross-links) indicate that it consists of one chain from the COL2 domain of the type IX collagen molecule that carries N-telopeptides from type II collagen covalently linked to a hydroxylysine residue somewhere along it.

These conclusions imply that individual type IX collagen molecules function in cartilage matrix in covalent linkage to type II collagen molecules probably located on the surface of fibrils. Indeed, immunogold electronmicroscopic localization has shown that type IX collagen decorates the surface of type II collagen fibrils in chick cartilage, particularly where fibrils intersect [22]. Having at least two sites for cross-linking to type II collagen, the type IX collagen molecule has the potential, therefore, to bridge fibrils covalently. If it so functions, type IX collagen could profoundly influence the material and biological properties of cartilage. It may act as a 'fusible link' that is cleavable by proteases (or perhaps mechanical forces) to permit normal growth and remodelling, and possibly to initiate the swelling and lost cohesiveness of articular cartilage seen in osteoarthritis.

If bovine type IX collagen is a type of proteoglycan as it is in the chick [8–11], the glycosaminoglycan chains may have the function of targeting the molecule to the correct sites for cross-linking on type II fibril surfaces. It is also possible, however, that the principal function of type IX collagen is to adapt the physical properties of type II fibril surfaces to their intimate enmeshment with proteoglycans.

ACKNOWLEDGEMENTS

This word was supported by grants from the US PHS National Institutes of Health, AR36794, AR37318 and GM15731.

NOTE ADDED IN PROOF

The first eight residues of the bovine type IX chain shown in fig. 4c match exactly a sequence recently reported for this domain of the chicken α2(IX) chain [McCormick et al. (1987) Proc. Natl. Acad. Sci. USA 84, 4044–4048].

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